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Multiplex PCR for detecting EHEC infections

The present invention originates from the diagnostic area of the detection of hemorrhagic diarrheal diseases.

Enterohemorrhagic E. coli pathogens (EHEC) are dangerous pathogens of diarrheal diseases which may be transmitted both by foodstuffs and by smear infections. Enterohemorrhagic E. coli organisms are able to form highly potent cytotoxins. These are proteins which have great similarity with the Shiga toxin of Shigella dysenteriae type 1 and are therefore called Shiga toxin 1 and Shiga toxin 2. The genes coding therefor for the respective subunits A and B are referred to as stxA1 GenBank number M19473) and stxB1 GenBank number M19473) and stxA2 (GenBank number X07865) and stxB2 GenBank number X07865). Pathogenic E. coli strains may contain either one of the two Shiga toxin genes or else both. In addition, the pathogens may have further associated virulence factors such as EHEC intimin, EHEC hemolysin, EHEC catalase, EHEC serine protease and EHEC enterotoxin.

The diagnostic detection of EHEC is, because of the transmission routes and of the low infectious dose of about 10^2-10^3 organisms, important not only for those with acute disease but also in order to identify possible excretors or find other sources of infection. In the state of the art,EHEC

infections are detected by microbiological means using sorbitol McConkey selective agar plates or by means of toxin ELISAs. In addition, PCR detection methods exist and can be used to detect Shiga toxin gene sequences (for example Chen et al., Applied and Environmental Microbiology Vol. 64 No. 11, pp. 4210-4116, 1998; Gannon et al., Applied Environmental Microbiology, Vol. 58 No. 12, pp. 3809-3815, 1992; Pierard et al., Journal of Clinical Microbiology Vol. 36, No. 11, pp. 3317-3322,). However, the only methods currently used in routine diagnosis are those which amplify the sequence coding for the B subunit.

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Sequence analysis of the Shiga toxin genes from various isolates has shown that not only are the primary sequences of Shiga toxin 1 and Shiga toxin 2 different from one another, but that, in addition, different EHEC isolates in which Shiga toxin 2 can be detected immunologically also display different alleles in relation to the sequence of the corresponding gene. Thus, the only primer sequences suitable for diagnostic detection of Shiga toxin 2 are those which are directed against highly conserved regions within the Shiga toxin genes and thus are able to detect the sequences of all the alleles.

In addition to the classical diarrheal diseases caused by known human-pathogenic EHEC pathogens, there exist other diarrheal diseases with a similar clinical picture. It has likewise been possible to diagnose enterohemorrhagic pathogens as the cause of these diseases by tests with Vero cell cultures (Pierard et al., Lancet 338, p. 762, 1991;

This object is achieved by a nucleic acid

amplification reaction for the detection of clinically relevant EHEC infections, with which it is possible simultaneously to identify stxA1 and stxA2 sequences which are derived both from human-pathogenic and from swine-p athogenic pathogens.

The invention thus relates to a method with which, in a multiplex amplification reaction for the detection of clinically relevant EHEC infections, both stxA1 and stxA2 sequences are amplified, and which is characterized in particular by amplification both of human-pathogenic stxA2 isoforms and of swine-pathogenic $stxA2_e$ isoforms. In this connection, the term "multiplex amplification reaction" refers to PCR methods in which at least two different primer pairs are used, one primer pair being used to amplify stx1 sequences and a second primer pair being used to amplify stx2 sequences. The term "swine pathogen" is used within the scope of this application for pathogens which have a Shiga toxin gene $stx2_e$ (Weinstein et al., J. Bacteriol. 170, pp. 4223-4230, 1988), GenBank number: M21534) and primarily cause edema disease in swine, but may also lead to diarrheal diseases and extraintestinal disease manifestations in humans.

Primers which have proved particularly suitable for carrying out the method of the invention have a length of 17-25 nucleotides, whose sequences is either identical to a

sequence as shown in SEQ ID No. 1-4, whose sequences represent continuous part-sequences of one of the sequences as shown in SEQ ID No. 1-4, or in which a sequence as shown in SEQ ID No. 1-4 forms a continuous part-sequence of the primer. The use of one, preferably more than one, particularly preferably all, of the primers of the invention for detecting EHEC infections is likewise an aspect of the invention.

Thus, multiplex amplification reactions in which either one, more than one or all of the primers of the invention are used have proved advantageous for detection of clinically relevant EHEC infections.

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The primers ordinarily used are chemically synthesized deoxyribonucleotides. However, it is also possible in principle to employ other nucleic acid molecules or their derivatives such as, for example, PNA (peptide nucleic acids). In addition, primers of the invention can also be conjugated to detectable or immobilizable molecules.

In a specific embodiment of the method of the invention, the product of the amplification is, in order to increase the sensitivity of the assay, additionally detected by hybridization. The hybridization probes suitable for this purpose preferably have a length of 25-35 nucleotides. Chemically synthesized deoxyribonucleotides are likewise ordinarily used, but may optionally be replaced by other nucleic acid molecules or their derivatives such as, for example, PNA (peptide nucleic acids). By definition, these probes are conjugated to a detectable label. This may be, for example, a fluorescent dye, an enzyme, a radioactive atom or a group detectable by mass spectrometry.

The invention therefore likewise relates to hybridization probes having a sequence or part-sequence as shown in SEQ ID No. 5-8. However, it has proved to be advantageous if a hybridization probe has a sequence which is identical or complementary to a region of the stxA1 gene or of the stxa2 gene which corresponds to the enzymatically active site of the polypeptide chain encoded by these genes. In the sequence of stxA2, this active site is located at nucleotide position 803-805 (Jackson et al, J. Bacteriol. 172, pp. 3346-3350, 1990). Hybridization probes having a sequence or part-sequence as shown in SEQ ID No. 8 are thus particularly preferred.

In a further particular embodiment, the multiplex amplification products obtained according to the invention are detected by means of fluorescence detection. Given the choice of a suitable fluorescent agent, it can be added even to the PCR mixture without impairing the amplification efficiency. This can take place, for example, by carrying out the PCR reaction in the presence of a fluorescent compound which, on binding to double-stranded DNA molecules and on excitation with light of a suitable wavelength, emits fluorescent signals (WO 97/46707):

The invention thus also relates to a method in which the multiplex amplification products are detected with the aid of a compound which fluoresces on binding to double-stranded DNA. For example, the fluorescent dye SybrGreen can be employed for a method of this type (WO 97/46714).

The present invention further relates to a method in which the stx sequences are detected with the aid of one or more fluorescence-labeled hybridization probes. Various embodiments are possible in this case, such as, for example, the use of molecular beacons (WO 95/13399, US patent No. 5 118 801) or so-called TaqMan probes (WO 96/34983).

Also suitable for the quantitative detection of nucleic acids are hybridization probes labeled with fluorescent dyes, such as, for example, oligonucleotides whose binding to a nucleic acid target can be detected by the principle of fluorescence resonance electron transfer (FRET) (WO 97/46707). This entails a so-called donor component, for example fluorescein, being excited with light of a particular wavelength. If a suitable acceptor component, such as, for example, certain rhodamine derivatives, is in the proximity, then resonance energy transfer to the acceptor component takes place, so that the acceptor molecule emits light of a particular emission wavelength.

The hybridization probes can be labeled by standard methods at the 5′ end, at the 3′ end or else internally. In a preferred embodiment, the various dyes are bound to two different hybridization probes which are able to hybridize in proximity onto the target nucleic acid. When in this embodiment both hybridization probes are bound to the target DNA, then both components of the FRET system are also in mutual proximity, so that fluorescence resonance energy transfer can be measured. This makes indirect quantification

of the target DNA possible.

The two oligonucleotide probes can moreover hybridize onto the same strand of the target nucleic acid, in which case one dye is preferably located on the 3'-terminal nucleotide of the first probe, and the other dye is preferably located on the 5'-terminal nucleotide of the second probe, so that the distance between the two is only a small number of nucleotides, and this number can be between 0 and 30. On use of fluorescein in combination with a rhodamine derivative such as, for example, LC-RED 640 or LC-RED 705 (Roche Molecular Biochemicals) it has emerged that the distances are advantageously from 0-15, in particular 1-5, nucleotides and, in many cases, one nucleotide. While maintaining the nucleotide distances between the dye components it is also possible to use probes which are conjugated not terminally but internally to one of the dyes. In the case of double-stranded target nucleic acids it is also possible to employ probes which bind to different strands of the target, as long as a particular nucleotide distance of 0 to 30 nucleotides is maintained between the two dye components.

Methods of the invention which have accordingly proved to be particularly advantageous are those in which stxA1 and stxA2 are detected with the aid of fluorescence resonance energy transfer. The invention likewise relates to hybridization probes having a sequence or part-sequence as shown in SEQ ID No. 5-8 and to methods in which these

specific hybridization probes are employed for the detection of clinically relevant EHEC infections.

A specific embodiment of the invention is thus also represented by fluorescence-labeled probe pairs either as shown in SEQ ID No. 5 and 6 or as shown in SEQ ID No. 7 and 8, which are advantageously labeled with, in each case, a FRET donor component such as, for example, fluorescein and with a FRET acceptor component such as, for example, Cy5, LC-RED 640, LC-RED 705 or another rhodamine derivative. Correspondingly labeled oligonucleotide combinations are referred to hereinafter as "FRET pairs".

It has proved particularly advantageous to use such FRET pairs for detecting amplification products during or after a multiplex amplification reaction. In a particular embodiment, one of the two amplification primers can at the same time be labeled with one of the two dyes employed, and thus contribute one of the two components of the FRET.

The use of suitable FRET pairs for detecting multiplex amplification products makes parallel, so-called real-time monitoring of PCR reactions possible, it being possible to find data for generating the amplification product as a function of the number of reaction cycles completed. This usually takes place by the oligonucleotides of the FRET pair also hybridizing onto the target nucleic acid because of the reaction and temperature conditions during the necessary annealing of the amplification primers onto the nucleic acid to be detected, and an appropriately

product as a function of the number of reaction cycles completed. This usually takes place by the oligonucleotides of the FRET pair also hybridizing onto the target nucleic acid because of the reaction and temperature conditions during the necessary annealing of the amplification primers onto the nucleic acid to be detected, and an appropriately

measurable fluorescence signal being emitted with suitable excitation. It is thus possible on the basis of the data obtained to determine quantitatively the amount of target nucleic acid originally employed.

In another, preferred embodiment, the multiplex amplification products are detected after completion of the amplification reaction, in which case, after hybridization of the FRET pair onto the target nucleic acid to be detected, the temperature is increased continuously in a melting curve analysis. At the same time, the emitted fluorescence is measured as a function of the temperature and, in this way, a melting temperature at which the FRET pair employed no longer hybridizes onto the sequence to be detected is determined. If there are mismatches between the FRET pair employed and the amplification product, the melting point is significantly depressed. It is possible in this way to identify with one FRET pair different target nucleic acids whose sequences differ from one another slightly through one or a few point mutations.

This principle is employed according to the invention in a multiplex amplification reaction for detecting EHEC infections, in which there is use of an internal standard which differs from the stxA1 or stxA2 wild-type sequence (GeneBank number X07865) only in one or two point mutations. It is thus possible to distinguish amplified target nucleic acid and amplified internal standard from one another with

the aid of a melting curve analysis.

In this case, the standard is preferably employed only in small amounts of about 100 plasmid copies (1.7×10^{-22} mol), so that a positive signal relating to amplification of the internal standard not only indicates that the PCR has not been inhibited in any way in the particular mixture, but also represents a check of the sensitivity of the reaction.

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A further aspect of the method of the invention relates to distinguishing human-pathogenic stxA2 and swine-pathogenic $stxA2_e$ with the aid of the described melting curve analysis.

The use of FRET pairs as shown in SEQ ID No. 5 and 6 or 7 and 8 for determining melting curves or for distinguishing human-pathogenic stx and swine-pathogenic stx is in this connection likewise an aspect of the invention. This preferably entails use of a FRET pair as shown in SEQ ID No. 7 and 8.

The present invention additionally relates to kits which comprise various reagents for carrying out the methods of the invention. Such kits of the invention usually comprise amplification primers for carrying out a multiplex PCR as shown in SEQ ID No. 1-4. These kits may preferably also comprise hybridization probes, for example with sequences as shown in SEQ ID No. 5-8.

Furthermore, these kits may additionally comprise according to the invention primers and hybridization probes for amplification of DNA of one or more additional EHEC

virulence factors such as, for example, EHEC intimin, EHEC hemolysin, EHEC catalase, EHEC serine protease and EHEC enterotoxin. Finally, all the kits of the invention may additionally comprise reagents which are generally suitable for carrying out nucleic acid amplification reactions. These are preferably, but not exclusively, special buffers, Taq polymerase and deoxyribonucleotides.

Brief description of the figures:

Figures 1 and 2 show a melting curve analysis as described in example 2. The first derivative of the measured fluorescence is in each case depicted as a function of the respective temperature, measured with a FRET pair composed of fluorescein and LC-RED 640 for detecting stxA1 (figure 1) and a FRET pair composed of fluorescein and LC-RED 705 for detecting stxA2 (figure 2).

Example 1: DNA isolation from bacterial cultures and stool samples

Bacterial cultures were worked up after overnight culture in TSB broth (casein peptone, pancreatin digest 17.0 g/l; soy meal peptone, papain digest 3.0 g/l; sodium chloride 5.0 g/l; dipotassium hydrogen phosphate 2.5 g/l; glucose 2.5 g/l) with the aid of a commercial DNA extraction method (QIAamp DNA Mini Kit, Qiagen, Catalog No. 51304). For this purpose, 200 µl of the bacterial suspension were incubated with 20 µl of proteinase K and 200 µl of ATL buffer

at 56°C for 10 min. 200 µl of 96% ethanol were then admixed with the suspension. The solution was then put onto a QIAamp spin column and centrifuged at 6 000 g for 1 min. The columns were then washed once with 500 µl each of AW1 and AW2 buffers (bench centrifuge 20 000 g). After the second washing step, the column was centrifuged until dry once. The purified DNA was subsequently eluted with 200 µl of AE buffer (10 mM Tris/HCC 0.5 m MEDT A pH 9.0). Before use in the PCR reaction, the DNA concentration and purity were determined in a photometer (spectrum from 260 nm to 320 nm). A maximum of 500 ng of template DNA were employed for each PCR mixture. In background investigations, a DNA equivalent to 10^7 bacteria (corresponds to about 55 ng of DNA) was employed.

PCR investigations were carried out directly on stool samples using a special stool kit (QIAamp DNA Stool Kit, containing the same buffers as the QIAamp DNA Mini Kit). For this purpose, 200 mg of stool (200 µl in the case of diarrheal stools) were thoroughly mixed with 600 µl of ASL buffer. In parallel with this, 300 mg of matrix AX (adsorbent for inhibitors in stool samples) are resuspended in 900 µl of the same buffer. This suspension was then added to the dissolved stool sample and thoroughly mixed. Subsequently, the homogenate was incubated at 70°C for 5 min. The matrix AX and undissolved stool particles were then pelleted by a centrifugation step at 20 000 g for 3 min. 200 µl of the supernatant were then mixed in analogy to the above-mentioned QIAamp DNA mini protocol with 20 µl of proteinase K and

likewise incubated at 56°C for 10 min. Subsequently, entirely in analogy to the procedure for bacterial cultures, using the same buffers, the DNA was bound to a QIAamp spin column, washed, eluted and measured in a photometer. The same amounts of DNA as described above were employed in the subsequent PCR reaction.

Example 2: Amplification and identification of the amplification products

A multiplex PCR for detecting stxA1 and stxA2 in DNA isolated by one of the methods of example 1 was carried out in the LightCycler system (Roche Molecular Biochemicals) in accordance with the manufacturer's information. The amplification product was detected according to two different protocols either with the aid of SybrGreen as double-stranded DNA-binding agent or, alternatively, with the aid of FRET hybridization probes.

All the primers and hybridization probes used were HPLC-purified and were stored in stock solutions of 100 pM/µl (primers) or 3 pM/µl (probes). The primers employed were selected in this case so that it was possible to amplify a 418 bp fragment of stxA1 (nucleotide position 598-1015, primers as shown in SEQ ID No. 1 and 2) and a 264 bp fragment of stxA2 (nucleotide position 679-942, primers as shown in SEQ ID No. 3 and 4).

The hybridization probes employed for the detection were labeled by standard protocols. For detecting stxA1, an

oligonucleotide as shown in SEQ ID No. 5 was labeled at the 3' end with fluorescein and an oligonucleotide as shown in SEQ ID No. 6 was labeled at the 5' end. An oligonucleotide as shown in SEQ ID No. 7 was labeled at the 3' end with fluorescein and an oligonucleotide as shown in SEQ ID No. 8 was labeled at the 5' end with LC-RED 705 as hybridization probes for detecting stxA2.

Detection with SybrGreen:

2.0 µl of DNA master SYBR Green I (Roche Molecular Biochemicals, containing buffers, Taq DNA polymerase, dNTPs, $MgCl_2$ and SYBR Green I dye)

10 pM of each primer employed as shown in SEQ ID No. 1-4

2.4 µl of 25 mM $MgCl_2$ (working concentration 4 mM)

10 µl of DNA

20 µl complete mixture

Detection using hybridization probes:

2.0 µl of DNA master for hybridization probes (Roche Molecular Biochemicals, containing buffers, Taq polymerase, dNTPs and $MgCl_2$)

10 pM of each primer employed as shown in SEQ ID No. 1-4

3 pM of each hybridization probe employed as shown in SEQ ID No. 5 and 6 for stxA1 of SEQ ID No. 7 and 8 stxA2

2.4 µl of 25 mM $MgCl_2$ (final concentration 4 mM)

8.0 µl of DNA

20 µl complete mixture

Both mixtures were run with the same PCR program and terminated with a melting curve:

Temperature cycles:

95°C 120 sec denaturation at the start of the program

95°C 1 sec

55°C 5 sec (touchdown from 60°C to 55°C in 5 steps of 1°C)

72°C 20 sec

45 cycles

The melting curves were constructed after previous brief denaturation at 95°C in an interval from 50°C to 95°C in 0.2°C steps with continuous fluorescence measurement on channel 1 (SYBR Green), channel 2 (LC-Red 640) or channel 3 (LC-Red 705) with the aid of the LightCycler software 3.0.

In SYBR Green mode, the specificity of PCR products from stool samples was found via the melting point compared with a control of stx-positive bacteria, in particular in order to be able to distinguish the amplification product from nonspecific primer dimers. In addition, all the results were verified by gel electrophoresis.

In the case of the FRET hybridization probe format it was necessary to employ the color compensation file of the LightCycler 3.0 software to avoid crosstalk effects between channel 2 and 3. The results of a typical experiment are disclosed in figure 1 and 2:

Figure 1 shows the temperature dependence of the fluorescence from mixtures with different initial DNA concentrations in channel 2 (LC-RED 640) for detecting stxA1; figure 2 shows the fluorescence of the same samples measured in channel 3 (LC-RED 705) for detecting stxA2. In each case,

the first derivative of the fluorescence measured as a function of the particular melting curve temperature in accordance with the information from the LightCycler manufacturer is depicted. The temperatures of the curve maxima found thus correspond to the melting points of the respective hybridization probes.

Example 3: Sensitivity

DNA of the stx1 and stx2-positive E. coli strain EDL 933 was extracted and quantified by photometry as in example 1. Based on the assumption that 2×10^8 bacteria contain about 1 µg of DNA, the number of bacteria worked up was inferred and serial dilutions were set up. Cultured stool samples from routine diagnoses, which were free of intestinal pathogens, were processed by the method described above, as background. It was in this case possible to detect in multiplex mixtures as in example 2 equivalents of at least about 1.8 stx-positive bacteria in a background of about 1×10^7 stx-deficient bacteria in a reaction mixture.

Example 4: Specificity - detection of human- and swine-pathogenic EHEC

48 human isolates of various serotypes, whose genotype was unknown at the time of the invention but which had already been characterized as EHEC strains by other, prior art methods, were investigated as in example 2 in the FRET hybridization probe mode. In addition, 3 isolates from

pigs with E. coli edema disease were investigated. The result is shown in table 1:

Table 1: Detection of human- and swine-pathogenic stx

Serial No.	Code No.	Source	Serotype	*stx*$_1$	*stx*$_2$	Tm *stxA2*
1	485/98	CI	O145:H$^-$	+	-	-
2	531/98	CI	O145:H$^-$	+	-	-
3	563/98	CI	O113:HNT	+	-	-
4	633/98	CI	O26:H$^-$	-	+	72°C
5	741/98	CI	O121:H$^-$	-	+	72°C
6	742/98	CI	O8:H$^-$	-	+	63°C
7	768/98	CI	O30:H21	-	+	72°C
8	802/98	CI	O157:H$^-$	-	+	72°C
9	1115/98	CI	O157:H$^-$	+	+	72°C
10	1168/98	CI	O128:H$^-$	+	+	63°C
11	1211/98	CI	O60:H$^-$	-	+	63°C
12	1244/98	CI	O6:H8	-	+	72°C
13	1273/98	CI	O6:H8	-	+	72°C
14	1295/98	CI	ONT:H$^-$	-	+	72°C
15	1306/98	CI	O157:H$^-$	+	+	72°C
16	1568/98	CI	O103:H$^-$	+	-	-
17	1613/98	CI	ONT:HNT	+	-	-
18	1695/98	CI	O103:H$^-$	+	-	-
19	1760/98	CI	O129:H$^-$	+	+	63°C
20	1762/98	CI	O129:H$^-$	+	+	63°C
21	1771/98	CI	O113:H2	+	+	63°C
22	54/99	CI	O103:H18	+	-	-
23	90/99	CI	O157:H$^-$	-	+	72°C
24	109/99	CI	O157:H$^-$	-	+	72°C
25	143/99	CI	O92:H32	-	+	63°C
26	144/99	CI	O92:H32	-	+	63°C
27	159/99	CI	O76:H$^-$	+	+	63°C
28	197/99	CI	O128:HNT	+	+	63°C
29	209/99	CI	ONT:HNT	-	+	72°C
30	240/99	CI	O30:HNT	-	+	72°C
31	285/99	CI	O145:HNT	+	-	-
32	363/99	CI	ONT:H$^-$	+	+	63°C
33	497/99	CI	O103:H4	+	-	-
34	516/99	CI	ONT:H$^-$	+	-	-

Table 1: Detection of human- and swine-pathogenic stx

Serial No.	Code No.	Source	Serotype	*stx*$_1$	*stx*$_2$	Tm *stxA2*
35	575/99	CI	O157:H$^-$	-	+	72°C
36	576/99	CI	O157:H$^-$	-	+	72°C
37	594/99	CI	ONT:H$^-$	+	-	-
38	649/99	CI	ONT:H9	-	+	72°C
39	680/99	CI	O157:H$^-$	+	+	72°C
40	707/99	CI	ONT:HNT	+	+	63°C
41	713/99	CI	O115:H10	+	-	-
42	720/99	CI	O111:H$^-$	+	-	-
43	789/99	CI	O103:HNT	+	-	-
44	791/99	CI	O91:HNT	+	-	-
45	809/99	CI	ONT:HNT	+	-	-
46	826/99	CI	ONT:HNT	+	-	-
47	827/99	CI	ONT:HNT	-	+	72°C
48	834/99	CI	ONT:HNT	+	-	-
49	A 3473-1/98	ED	O139:H1	-	+	63°C
50	A 3621-2/98	ED	O139:H1	-	+	63°C
51	82812/99	ED	O139:H1	-	+	63°C

CI = clinical isolate

Ed = edema disease

It was in this case possible to identify all the human isolates as stx-positive, with detection only of stx1 in 18 strains, only of stx2 in 19 strains and of both genes in 11 strains. The three pig isolates were likewise stx2-positive.

On use of the hybridization samples of the invention as shown in SEQ ID No. 7 and 8, differences in the melting temperatures of stxA2 were measured in different isolates: melting temperatures of 71-72°C were found for stxA2 in amplicons of 18 of the 30 stxA2-containing human isolates. This temperature is identical to the Tm found in preceding

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the DNA of the remaining 12 stxA2-containing human isolates and for the DNA from the three swine-pathogenic strains, which certainly contain the $stx2_e$ allele. It can be concluded from the identical Tm that the 12 human-pathogenic isolates are attributable to swine-pathogenic EHEC strains and presumably likewise contain the $stx2_e$ allele. This supposition was confirmed by sequence analysis of the PCR products from the corresponding 12 isolates.

Overall, this example shows that both human-pathogenic and swine-pathogenic EHEC pathogens can be identified with the aid of the method of the invention.

Example 5: Specificity - avoidance of false-positive results

Specificity tests were carried out on 32 stx-negative bacterial strains listed in table 1. For this purpose, DNA was extracted as in example 1 from appropriate overnight cultures. The isolated DNA was subsequently investigated as in example 2 for the presence of stxA1 and stxA2 using the SybrGreen mode. The result was always unambiguously negative. As inhibition control, the DNA was mixed with DNA of the stx1- and stsx2-positive E. coli strain EDL 933 in a parallel mixture and tested for stx1 and stx2 in the same run, unambiguously positive signals being obtained without exception.

Table 2: Bacterial isolates tested in the specificity test	
Aeromonas hydrophilia	CLINICAL ISOLATE
Bacillus subtilis	ATCC 6633
Bacillus subtilis	ATCC 6051
Campylobacter coli	CLINICAL ISOLATE
Campylobacter jejuni	ATCC 33560
Candida albicans	ATCC 10231 Type 3
Citrobacter freundii	CLINICAL ISOLATE
Enterobacter cloacae	CLINICAL ISOLATE
Enterococcus faecalis	ATCC 10541
Enterococcus faecalis	CLINICAL ISOLATE
Enterococcus faecium	ATCC 19434
Enterococcus faecium	CLINICAL ISOLATE
Escherichia coli	ATCC 25922
EAEC O-42	CLINICAL ISOLATE
EIEC 460858	CLINICAL ISOLATE
EPEC E 2348/69	CLINICAL ISOLATE
ETEC	CLINICAL ISOLATE
Helicobacter pylori	CLINICAL ISOLATE
Klebsiella pneumoniae	ATCC 10031
Morganella morganii	CLINICAL ISOLATE
Plesiomonas shigelloides	CLINICAL ISOLATE
Proteus mirabilis	ATCC 1453
Proteus vulgaris	CLINICAL ISOLATE
Pasteurella canis	CLINICAL ISOLATE
Pseudomonas aeruginosa	ATCC 27853
Salmonella enteritidis	CLINICAL ISOLATE
Salmonella typhimurium	CLINICAL ISOLATE
Shigella flexneri	CLINICAL ISOLATE
Staphylococcus aureus	CLINICAL ISOLATE
Staphylococcus epidermidis	ATCC 12228
Streptococcus agalactiae	CLINICAL ISOLATE
Yersinia enterocolitica	CLINICAL ISOLATE

This example thus shows that the method of the invention is suitable for specific detection of EHEC infections.